

Uncoupling of *Chlamydomonas* Flagellar Gene Expression and Outgrowth from Flagellar Excision by Manipulation of Ca^{2+}

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Abstract. *Chlamydomonas* cells respond to certain environmental stimuli by shedding their flagella. Flagellar loss induces a rapid, transient increase in expression of a specific set of genes encoding flagellar proteins, and assembly of a new flagellar pair. While flagellar gene expression and initiation of flagellar outgrowth are normally tightly coupled to flagellar excision, our results demonstrate that these processes can be uncoupled by manipulating Ca^{2+} levels or calmodulin activity. In our experiments, wild-type cells were stimulated to excise their flagella using mechanical shearing, and at times after deflagellation, flagellar lengths were measured and flagellar mRNA abundance changes were determined by S1 nuclease protection analysis. When extracellular Ca^{2+} was lowered by ad-

dition of EGTA to cultures before excision, flagellar mRNA abundance changes and flagellar outgrowth were temporally uncoupled from flagellar excision. When extracellular Ca^{2+} was lowered immediately after excision or when calmodulin activity was inhibited with W-7, flagellar outgrowth was uncoupled from flagellar excision and flagellar mRNA abundance changes. Whenever events in the process of flagellar regeneration were temporally uncoupled, the magnitude of the flagellar mRNA abundance change was reduced. These results suggest that flagellar gene expression may be regulated by multiple signals generated from these events, and implicate Ca^{2+} as a factor in the mechanisms controlling flagellar regeneration.

CELLULAR responses to environmental cues often involve complex interactions between signal transduction pathways, gene regulatory circuits, and changes in cell morphology. In many cases, these responses are integrated through an intracellular messenger, such as Ca^{2+} (5). Although the role of Ca^{2+} in signaling mechanisms, physiological responses, and cell movements has been well studied, relatively little is known about the role of Ca^{2+} in regulating gene expression. The flagellar regeneration system of the unicellular green alga *Chlamydomonas reinhardtii* serves as a key model for studying the complexities of these types of interactions. A variety of extracellular treatments cause *Chlamydomonas* to excise their flagella (19a, 19, 21, 32). When flagella are shed, expression of a specific set of flagellar genes, including tubulin, is stimulated (1, 9), and cells begin to assemble a new pair of full-length flagella, using flagellar proteins translated from these newly synthesized mRNA templates, and preexisting flagellar proteins from a pool of unassembled precursors (21).

The involvement of Ca^{2+} in several aspects of flagellar regeneration has been demonstrated in previous studies. For example, some studies suggest that flagellar excision itself requires Ca^{2+} (19a, 24 and references therein). In addition, outgrowth of flagella after excision is dependent on Ca^{2+} . Outgrowth is delayed or prevented by lowering the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_e$) to $<10^{-6}\text{M}$, and occurs when the $[\text{Ca}^{2+}]_e$ is restored (18, 23). In other studies, low $[\text{Ca}^{2+}]_e$ /high salt medium causes flagellated cells to resorb

and disassemble their flagella in the cytoplasm; upon readdition of Ca^{2+} to resorbed cells, flagellar outgrowth occurs, and flagellar protein synthesis is also induced (11). Because the effects of Ca^{2+} on flagellar gene expression at a control level preceding translation have not been examined, we began our studies by examining the effects of Ca^{2+} on flagellar mRNA abundances and the interdependence of flagellar outgrowth and flagellar gene expression.

Flagellar gene induction and flagellar outgrowth, the cellular responses that are the focus of this study, are normally tightly coupled to the stimulus of flagellar excision during flagellar regeneration. We report here that these responses can be temporally uncoupled from flagellar excision by manipulating the $[\text{Ca}^{2+}]_e$ or by inhibiting calmodulin activity with W-7, suggesting that the process of flagellar regeneration is composed of discrete, separable events that are regulated by Ca^{2+} . In addition, we find that the magnitude of the flagellar mRNA abundance change is reduced whenever events during flagellar regeneration are temporally uncoupled, suggesting that the normal flagellar mRNA abundance change is composed of several overlapping gene regulatory responses contributed by these events.

Materials and Methods

Cell Cultures

Chlamydomonas reinhardtii, wild-type strain 137c, mt(-), vegetative cells

were used in all experiments. Cells were cultured (25°C; 14 h light/10 h dark cycle) in medium I of Sager and Granick (22), with continuous aeration. Cultures were grown to a density of 5×10^5 – 4×10^6 cells/ml. Cell density was determined using a Neubauer counting chamber (Clay-Adams, Parsippany, NJ).

Deflagellation and Flagellar Length Measurements

Flagella were detached by mechanical shearing with a VirTis homogenizer (The VirTis Co., Inc., Gardiner, NY) (21). Treatments for 1 min. at a setting of 35 resulted in >99% deflagellation of control cells, and >95% deflagellation of cells in low Ca^{2+} -containing medium. To determine flagellar regeneration kinetics, samples of cells were fixed with 1% glutaraldehyde at specific times after deflagellation and random fields of cells were photographed with Kodak Technical Pan film using a Zeiss phase-contrast photomicroscope. The negatives were projected on a screen and the flagella measured with a flexible ruler. To aid in measuring, a micrometer was photographed at the same magnification as the cell samples and used to calibrate the ruler. Each reported length was the average of measurements on 36 randomly chosen cells. A zero was recorded for cells without flagella. The standard error of the mean was calculated for flagellar lengths at each time point.

General Experimental Procedure

Cells were harvested by low-speed centrifugation (400 *g* for 5 min) and resuspended in fresh medium to give a final concentration of 5×10^6 – 1×10^7 cells/ml. 1M HEPES-KOH, pH 7.5, was added to a final concentration of 8 mM in order to maintain a constant pH in the culture during the experiment. Control experiments demonstrated that HEPES buffer had no effect on flagellar excision, outgrowth, or mRNA abundance changes (data not shown), in agreement with other reports (14). Cells were allowed to recover from centrifugation for 1–2 h while stirring gently under constant illumination at room temperature. After recovery, the $[\text{Ca}^{2+}]_i$ was lowered by adding 1 M EGTA (NaOH), pH 7.5, immediately before or after deflagellation, so that the $[\text{Ca}^{2+}]_i$ was either low during deflagellation or low immediately after deflagellation. Before the cells were deflagellated and at specific times after deflagellation, cell samples were taken for RNA isolation, flagellar length measurements, and microscopic observation. At 120 min after deflagellation, 1 M CaCl_2 (NaOH), pH 8.0, was added in order to restore the $[\text{Ca}^{2+}]_i$ to normal ($\sim 3.6 \times 10^{-4}$ M). The initial pH of the culture, and the pH of the culture after addition of EGTA and CaCl_2 differed by at most 0.2 pH units in any experiment.

Cell viability was monitored closely during the initial 45 min after deflagellation by microscopic examination of the cells. Viable cells are easily distinguished by having highly refractile cell bodies when viewed with phase-contrast microscopy, and are able to regenerate flagella under permissive conditions (see Results). The cell bodies of dead or dying cells are dark when viewed with phase-contrast microscopy, and the cells do not regenerate flagella in permissive experimental conditions. Experimental trials in which >5% of cells were inviable 45 min after deflagellation were terminated.

When used, the calmodulin antagonists W-7 (Molecular Probes, Inc., Eugene, OR) or W-5 (Sigma Chemical Co., St. Louis, MO) (7) were added directly to cultures from a 10-mM aqueous stock. To wash out the W-7, cells were diluted with 2 vol of fresh medium lacking W-7, pelleted by low-speed centrifugation (400 *g* for 5 min), and resuspended in fresh medium.

$[\text{Ca}^{2+}]_i$ s were estimated using a computer program developed by Dr. R. Steinhardt which uses the methods of Portzehl et al. (16). However, due to the presence of 1.7 M sodium citrate, 3.7×10^{-4} M FeCl_2 , and 8 mM HEPES (KOH) in the medium, our reported $[\text{Ca}^{2+}]_i$ s are estimates.

RNA Isolation

Total RNA was extracted from 5×10^7 – 1×10^8 cells at specific times after flagellar excision using a modification of the procedure described by Wagner et al. (29). Each sample was spun at 800 *g* for 5 min at room temperature, and the cell pellet was resuspended in 2 ml of lysis buffer (25). After a 5-min incubation with constant mixing, sodium acetate, pH 5.0, was added to a final concentration of 0.2 M, an equal volume of 10 mM Tris/1 mM EDTA-saturated hot phenol (55°C) was added, and the mixture was emulsified. During the hot phenol extraction, samples were held in a 55°C water bath for 10 min with occasional mixing. The phases were separated and an equal volume of 50:50 phenol/chloroform was mixed with the aqueous phase. After separating the phases again, a final extraction with an equal volume of 96:4 chloroform/isoamyl alcohol was followed by two precipita-

tions with 2 M ammonium acetate and 2 vol of 100% ethanol. The ethanol precipitates were collected, washed with 70% ethanol, dried, and resuspended in distilled water. Roughly equivalent amounts of total RNA were obtained from each time point.

Preparation of Probes

To prepare 3' end-labeled probes, 10 μg each of cDNA clones encoding the deflagellation-induced $\beta 2$ -tubulin mRNA, pcf 8–31, and another deflagellation-induced mRNA, pcf3–21 (25), were digested with the restriction endonuclease BamHI. 10 μg of the cDNA clone encoding the constitutively expressed mRNA, pcf8–13 (25), was digested with BglII. The digested DNAs were treated with 12–16 U of Klenow (Promega Biotech, Madison, WI) and 80–100 μCi of α - ^{32}P -dATP (3,000 Ci/mmol; NEN Research Products, Boston, MA and Amersham Corp., Arlington Heights, IL) (13) and then extracted sequentially with an equal volume of phenol/chloroform (50:50) and chloroform/isoamyl alcohol (96:4). To separate unincorporated nucleotides from the labeled DNA probes, the samples were passed through Sephadex G-50 columns (Pharmacia Fine Chemicals Inc., Piscataway, NJ; obtained through Sigma Chemical Co.) (13). The specific activities of the probes used in all experiments were 2 – 6×10^6 dpm/ μg .

S1 Nuclease Protection Analysis

A modification of the S1 nuclease protection analysis by Weaver and Weissmann (30) was used to detect changes in mRNA levels of $\beta 2$ -tubulin, pcf3–21, and pcf8–13. Hybridization reactions containing 2.5–20 μg of total RNA, 0.02–0.04 μg of the appropriate probe, 4 μl of deionized formamide and 1 μl of hybridization buffer (30), were denatured for 15 min at 85°C and then incubated for 12–16 h at either 57°C (for $\beta 2$ -tubulin and pcf8–13 probes) or 53°C (for the pcf3–21 probe). 45 μl of ice-cold S1 buffer (0.15 M sodium acetate, pH 4.5, 1.25 M NaCl, 0.01 M ZnSO_4 and 25% glycerol) containing 150–200 U of the single-strand specific nuclease S1 (GIBCO BRL, Gaithersburg, MD) was added to each sample. After digestion at 25°C for 60 min, 0.5 $\mu\text{g}/\mu\text{l}$ of yeast tRNA, 10 mM EDTA, and 2.5 vol of 100% ethanol were added to precipitate each sample. The precipitates were collected, washed, dried, and resuspended in 3 μl of loading buffer each (0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA, 95% (vol/vol) deionized formamide). Each sample was boiled for 3 min, quick-chilled on ice, and then electrophoresed on a 7 M urea/7.5% polyacrylamide gel. The gel was dried and exposed to Kodak diagnostic x-ray film at room temperature.

The expected sizes of the $\beta 2$ -tubulin, pcf3–21, and pcf8–13 bands were 368, 400, and 376 bp, respectively. Although the coding regions of the $\beta 1$ -tubulin and $\beta 2$ -tubulin genes are highly homologous, the $\beta 2$ -tubulin probe does not detect $\beta 1$ -tubulin mRNA since the unique labeled site in the probe is in the 3'-untranslated region, which is significantly different between these two genes (33). Occasionally, a secondary product ~ 15 nt shorter than the major product was generated with the $\beta 2$ -tubulin and pcf3–21 probes. For the tubulin probe, this secondary product could result from S1 digestion at a less stable AT-rich site ~ 15 nt upstream from the 3' terminus of the transcript. The secondary product of the pcf3–21 reaction could be generated by a similar sequence. By comparing densitometric signals of experimental samples hybridized with dilutions of labeled probe, we estimate that our hybridization reactions contained >100-fold excess of probe over complementary RNA.

Densitometry

Band intensities from the S1 nuclease protection analyses were quantified either directly using a Betagen densitometer (Betagen Corp., Waltham, MA) or by transmission densitometry of band intensities on autoradiographs using a BioRad Model 620 Video Densitometer (BioRad Laboratories, Richmond, CA). For transmission densitometry, relative densities from several autoradiographic exposures were compared to insure uniformity in the measurements.

Results

Flagellar Outgrowth and Flagellar mRNA Abundance Changes in Normal Media Measured by S1 Nuclease Protection

To validate the use of S1 nuclease protection analysis in these

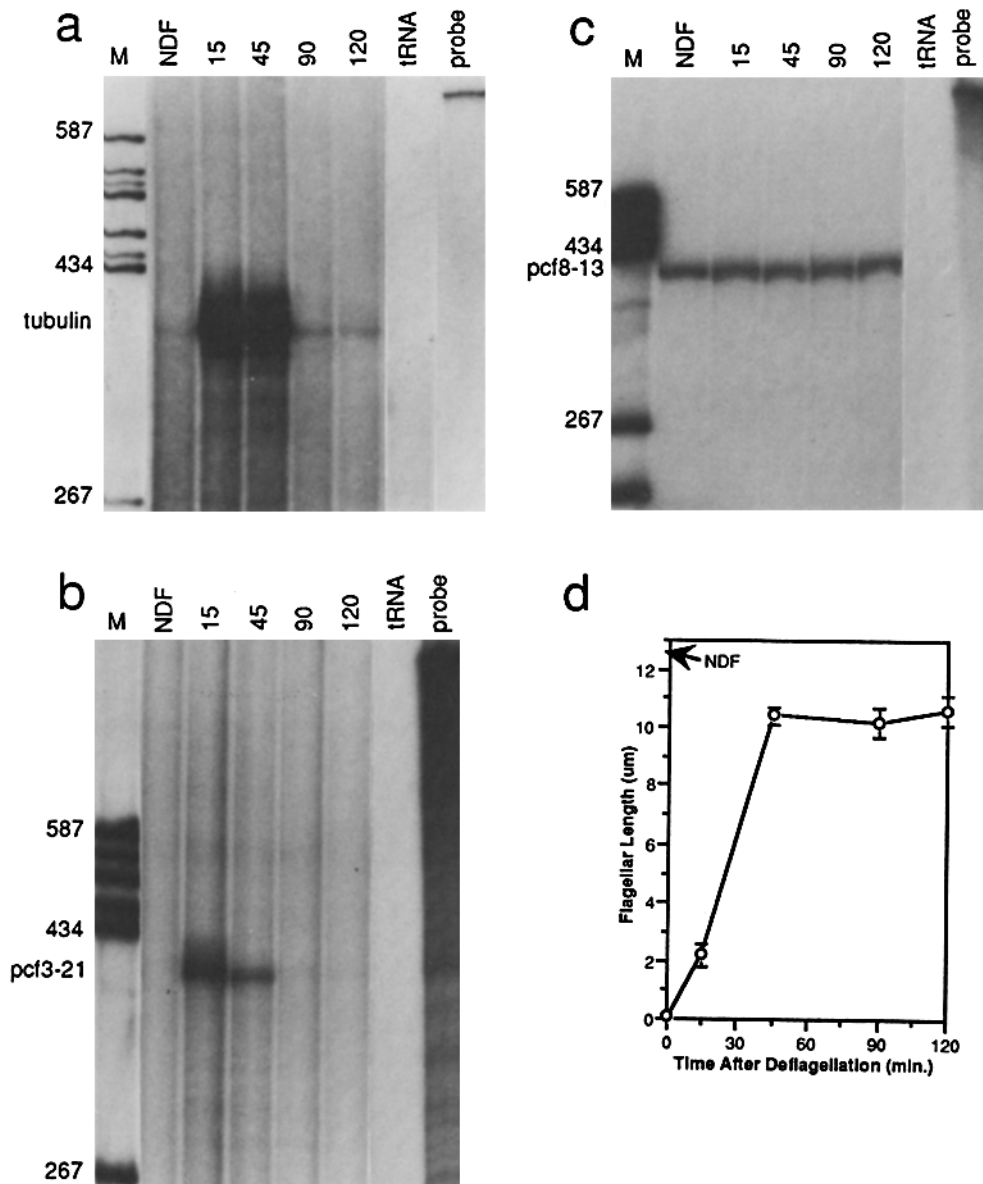


Figure 1. S1 nuclease protection analysis of flagellar mRNAs and flagellar outgrowth in *Chlamydomonas*. Autoradiographs show S1 nuclease protection analyses of (a) $\beta 2$ -tubulin, (b) pcf3-21, and (c) pcf8-13 mRNAs. For each panel, time after deflagellation is indicated in minutes above each lane. Positions of pBR322/HaeIII fragments are indicated (M, markers). Probe lane shows the migration of the full-length probe. Although only a portion of the total length of these gels is shown, no other bands were visible on the autoradiographs even after 4-6X longer exposures. (NDF, nondeflagellated cells.) (d) Kinetics of flagellar outgrowth after mechanical shearing in normal medium ($3.6 \times 10^{-4} M Ca^{2+}$). Each SEM is indicated with a vertical line. The flagellar length of nondeflagellated cells (SEM = 0.1) is indicated by the arrow.

studies, flagellar mRNA abundance changes following deflagellation in normal medium were investigated. The cDNA clones used, $\beta 2$ -tubulin and pcf3-21, contain sequences complementary to mRNAs that are induced by deflagellation (25). mRNA complementary to the other cDNA clone used, pcf8-13, is expressed at constant levels before and after deflagellation (25). Consistent with results of previous Northern blot and RNA dot analyses (25, 28), $\beta 2$ -tubulin and pcf3-21 mRNAs accumulated to high levels by 15 min after deflagellation (Fig. 1, a and b) compared to basal levels in nondeflagellated cells, remained elevated for the next 30 min, and then declined to pre-deflagellation levels by 120 minutes. pcf3-21 abundance returned to basal levels slightly earlier than $\beta 2$ -tubulin. Levels of the constitutively expressed mRNA, pcf8-13, remained unchanged before and after deflagellation (Fig. 1 c). Substitution of yeast tRNA for total RNA in the hybridization reaction showed that the concentration of S1 nuclease was sufficient to digest any unprotected

probe. The kinetics of flagellar outgrowth accompanying the mRNA abundance changes illustrated in Fig. 1 d were normal, in that highly synchronous flagellar outgrowth occurred with deceleratory kinetics beginning immediately after flagellar excision and reached 90% of the initial length by 90 min (21).

Flagellar mRNA Abundance Changes and Outgrowth Are Uncoupled from Flagellar Excision in Low Ca^{2+} -containing Media

Because a low $[Ca^{2+}]_i$ is known to affect the timing and extent of flagellar outgrowth (18), we examined whether a low $[Ca^{2+}]_i$ also affects flagellar mRNA abundance changes. Lowering the $[Ca^{2+}]_i$ to the $10^{-7} M$ range by addition of EGTA immediately before deflagellation (10-15 s before the homogenizer was turned on), so that the $[Ca^{2+}]_i$ was low during the deflagellation event, blocked an mRNA abun-

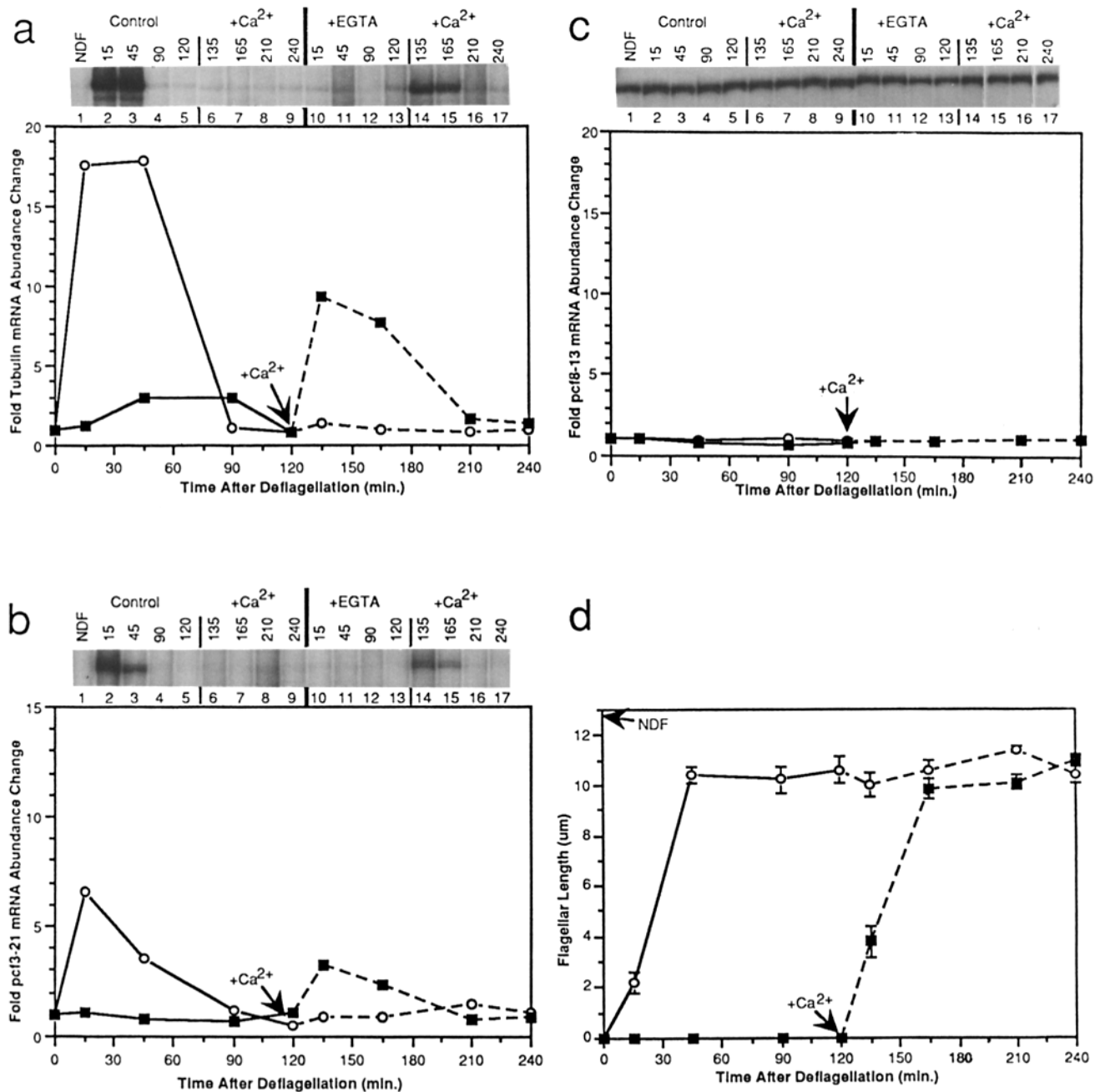


Figure 2. Flagellar mRNA abundance changes and the kinetics of flagellar outgrowth when the $[Ca^{2+}]_e$ was lowered to the 10^{-7} M range immediately before deflagellation. Autoradiographs show S1 nuclease protection analyses of (a) $\beta 2$ -tubulin, (b), pcf3-21, and (c) pcf8-13 mRNAs. For each panel, time after deflagellation is indicated in minutes above each lane. The graphs show relative changes in mRNA abundance over predeflagellation levels (predeflagellation level is 1). (NDF) nondeflagellated cells. (d) Kinetics of flagellar outgrowth when the $[Ca^{2+}]_e$ was lowered immediately before deflagellation. Each SEM is indicated with a vertical line. The flagellar length of nondeflagellated cells (SEM = 0.2) is indicated by the arrow. (open circles with solid lines) Cells deflagellated in normal medium; (open circles with dashed line) addition of Ca^{2+} 120 min after deflagellation in normal medium; (solid squares with solid line) $[Ca^{2+}]_e$ was lowered immediately before cells were deflagellated; (solid squares with dashed line) addition of Ca^{2+} 120 min after deflagellation in low Ca^{2+} -containing medium. In this experiment, EGTA was added to 0.4 mM to lower the $[Ca^{2+}]_e$, 0.8 mM $CaCl_2$ was added to restore the $[Ca^{2+}]_e$ to normal.

dance change until Ca^{2+} was restored to the culture. $\beta 2$ -Tubulin and pcf3-21 mRNAs remained at pre-deflagellation levels (Fig. 2, a and b, lanes 10-13) until Ca^{2+} was restored to the culture at 120 min after deflagellation. At this time, $\beta 2$ -tubulin and pcf3-21 mRNA abundances increased within 15 min after the addition of Ca^{2+} , and then returned

to predeflagellation levels within 90 min (lanes 14-17). The peaks of $\beta 2$ -tubulin and pcf3-21 mRNA abundance changes were only 50 and 42%, respectively, of abundance changes in control cells. Samples in lanes 6-9 demonstrate that the addition of Ca^{2+} at 120 min after deflagellation to cells in normal Ca^{2+} -containing medium did not induce a second

change in β 2-tubulin or pcf3-21 levels. pcf8-13 mRNA remained constitutively expressed throughout this experiment (Fig. 2 c).

Fig. 2 d shows the kinetics of flagellar outgrowth. The narrow SEMs calculated for each flagellar length measurement in this and all experiments demonstrate that our measurements of both flagellar outgrowth and mRNA abundance changes reflect the highly synchronous behavior of a population of viable cells. Only when Ca^{2+} was restored to normal did cells begin to assemble flagella, and with normal kinetics. The initiation of flagellar outgrowth was coincident with the observed submaximal increase in β 2-tubulin and pcf3-21 mRNA levels.

Cells were sensitive to deflagellation in extremely low $[\text{Ca}^{2+}]_e$, and it was often necessary to conduct several trial deflagellations at different $[\text{Ca}^{2+}]_e$ s within the 10^{-7}M range in order to determine conditions where both the kinetics of flagellar outgrowth were affected and cells remained viable. When lowering the $[\text{Ca}^{2+}]_e$ to the low 10^{-7}M range resulted in loss of cell viability, experiments were performed at a higher $[\text{Ca}^{2+}]_e$ still within the 10^{-7}M range where cell viability was not affected. At these higher $[\text{Ca}^{2+}]_e$ s, flagellar outgrowth and the peak of flagellar mRNA abundance were not blocked, but instead were delayed for 45–90 min after flagellar excision (data not shown). When cells from the same culture were treated with different $[\text{Ca}^{2+}]_e$ s, the duration of the delay before spontaneous flagellar outgrowth and mRNA abundance changes correlated with the extent of lowering the $[\text{Ca}^{2+}]_e$, in that lower $[\text{Ca}^{2+}]_e$ s resulted in longer delays before the initiation of flagellar outgrowth, and the lowest $[\text{Ca}^{2+}]_e$ s blocked both responses within the experimental time frame. Although the source of this variability has not been identified, several factors could contribute to the cells' response, including the age of the cultures or differences in the exact $[\text{Ca}^{2+}]_e$, since our reported $[\text{Ca}^{2+}]_e$ s are estimates (see Materials and Methods). However, these experiments indicate that there is a narrow window of 10^{-7}M Ca^{2+} in which changes in flagellar mRNA abundance and flagellar outgrowth are blocked.

These results indicate an uncoupling of flagellar outgrowth and flagellar mRNA abundance changes from flagellar excision in conditions of low extracellular Ca^{2+} . Also, they demonstrate for the first time that the magnitude of the flagellar mRNA abundance change can be affected (i.e., reduced), suggesting that the mRNA abundance change is not a fixed, "quantal" response, but may be a composite of several gene regulatory responses.

Flagellar Outgrowth Is Uncoupled from Flagellar Excision and mRNA Abundance Changes by Lowering Ca^{2+} after Deflagellation

When the $[\text{Ca}^{2+}]_e$ was lowered immediately after deflagellation (5–10 s after the homogenizer was turned off), flagellar outgrowth was blocked until Ca^{2+} was restored to the culture at 120 min after deflagellation (Fig. 3 d). Cells held in low Ca^{2+} -containing medium spontaneously initiated flagellar outgrowth between 120 and 135 min after deflagellation.

Submaximal β 2-tubulin and pcf3-21 mRNA abundance changes occurred with the same timing as in control cells (Fig. 3, a and b, lanes 6–9). Upon restoring normal Ca^{2+} conditions to half of the culture at 120 min, β 2-tubulin and pcf3-21 mRNA levels increased again within 15 min and

then declined to basal levels (lanes 14–17). An increase in β 2-tubulin and pcf3-21 mRNAs also occurred at 165 min after deflagellation in control cells held in low Ca^{2+} -containing medium (lanes 11–12). Although the timing and kinetics of the mRNA abundance changes in this experiment were normal, the magnitudes of β 2-tubulin and pcf3-21 abundance changes were submaximal compared to control cells. For β 2-tubulin, the initial peak after deflagellation, the peak that occurred upon Ca^{2+} addition, and the peak that was concomitant with the spontaneous initiation of flagellar regeneration were 74, 63, and 32%, respectively, of the peak in control cells after deflagellation in normal conditions. The corresponding peaks of pcf3-21 mRNA were 34, 50, and 14% those of control cells. RNA samples analyzed using probes for the α 1-tubulin and β 1-tubulin mRNAs showed similar mRNA abundance changes (data not shown). pcf8-13 mRNA remained constitutively expressed throughout this experiment.

These results demonstrate three important points. First, submaximal flagellar mRNA abundance changes occur after flagellar excision in the absence of flagellar outgrowth. While deflagellation in low Ca^{2+} blocked the flagellar mRNA abundance change (Fig. 2), lowering Ca^{2+} immediately after deflagellation permitted its occurrence, indicating that the mRNA abundance change requires Ca^{2+} . Second, as shown in Figs. 1–3, whenever flagella-less cells initiate flagellar outgrowth, a change in flagellar mRNA abundance occurs. Thus, while conditions exist where an mRNA abundance change occurs in the absence of flagellar outgrowth (Fig. 3), conditions have not been found where flagellar outgrowth initiates without an accompanying flagellar mRNA abundance change. Third, Ca^{2+} is required for flagellar assembly.

The Calmodulin Antagonist W-7 Uncouples Flagellar Outgrowth from Flagellar mRNA Abundance Changes after Deflagellation

To determine whether the Ca^{2+} requirement for flagellar outgrowth and flagellar mRNA abundance changes is mediated through calmodulin, the calmodulin antagonist W-7 was added to intact cells in normal Ca^{2+} -containing medium. Several different W-7 concentrations were tested to determine the optimal concentration for use in our experiments (Table I). Within 5–10 min after addition of 25 μM W-7, 60% of cells had lost their flagella. Microscopic examination of cell samples showed flagellated cells, cells with a single flagellum, flagella-less cells, and detached flagella in the field. This result is consistent with reports of differential sensitivity of flagella to different $[\text{Ca}^{2+}]_e$ s (8). 25 μM W-5, a much less potent analogue of W-7, did not cause the cells to excise their flagella. These data suggest that W-7 treatment alters intracellular Ca^{2+} homeostasis in a manner that ultimately results in flagellar excision.

Further experiments to determine the involvement of Ca^{2+} and calmodulin in flagellar outgrowth and mRNA abundance changes were performed by adding W-7 to cells just before deflagellation by mechanical shear. As shown in Fig. 4 d, W-7 reversibly blocks flagellar outgrowth. Cells deflagellated in the presence of W-7 did not initiate flagellar outgrowth until they were washed out of W-7-containing medium into normal medium. The kinetics of outgrowth after W-7 washout were similar to those of control cells

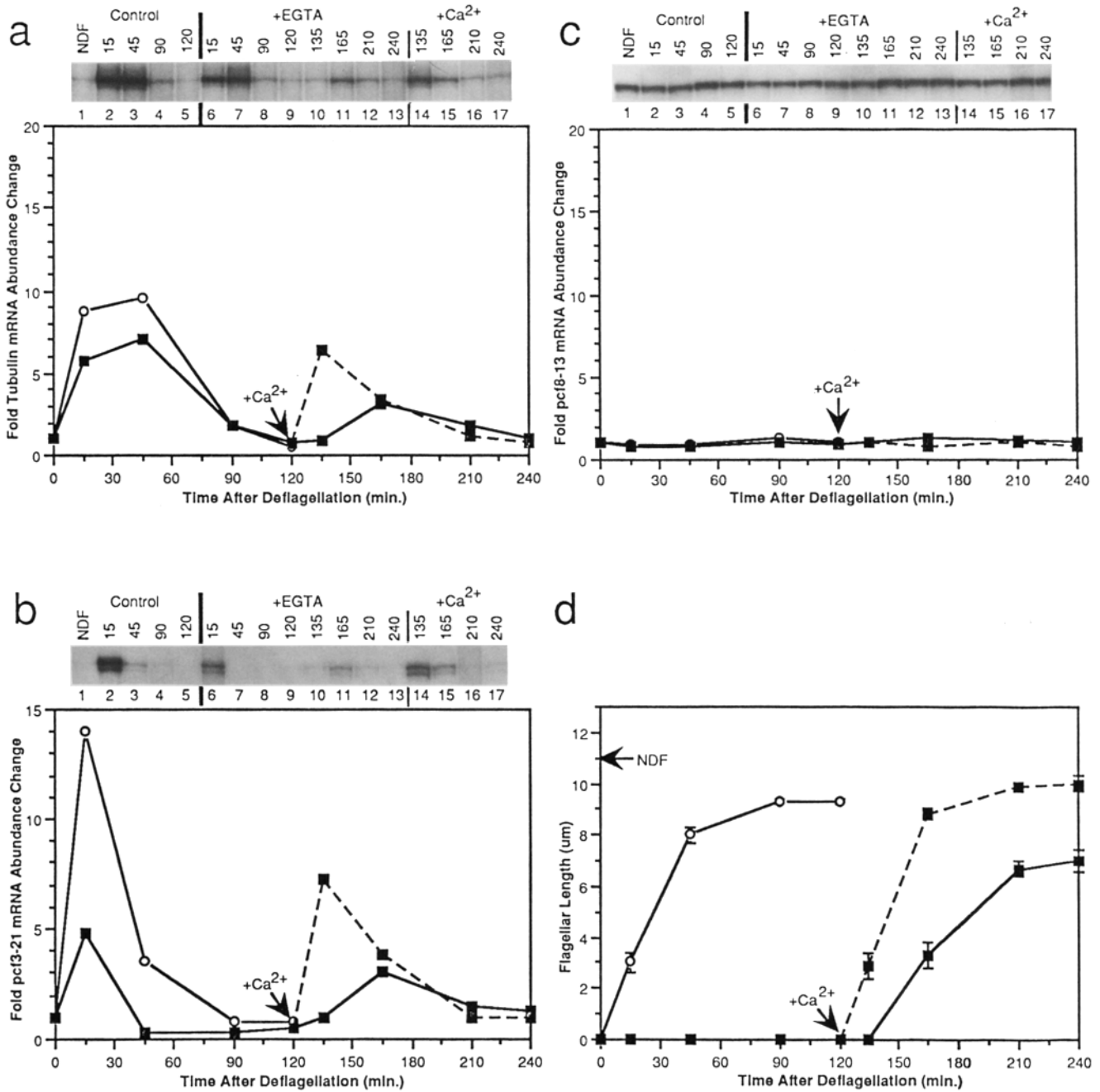


Figure 3. Flagellar mRNA abundance changes and the kinetics of flagellar outgrowth when the $[Ca^{2+}]_i$ was lowered to the 10^{-7} M range immediately after deflagellation. Autoradiographs show S1 nuclease protection analyses of (a) $\beta 2$ -tubulin, (b) pcf3-21, and (c) pcf8-13 mRNAs. For each panel, time after deflagellation is expressed in minutes above each lane. The graphs show relative changes in mRNA abundance over predeflagellation levels. (Predeflagellation level is 1.) (NDF) Nondeflagellated cells. (d) Kinetics of flagellar regeneration when the $[Ca^{2+}]_i$ was lowered immediately after deflagellation. Each SEM is indicated by a vertical line. The flagellar length of nondeflagellated cells (SEM = 0.2) is indicated by the arrow. (open circles with solid line) Cells deflagellated in normal medium; (solid squares with solid line) $[Ca^{2+}]_i$ was lowered immediately after cells were deflagellated; (solid squares with dashed line) addition of Ca^{2+} 120 min after deflagellation in low Ca^{2+} -containing medium. In this experiment, EGTA was added to 2.3 mM; 0.3 mM $CaCl_2$ was added to restore the $[Ca^{2+}]_i$ to normal.

Figure 4. Flagellar mRNA abundance changes and the kinetics of flagellar outgrowth when cells were deflagellated in the presence of the calmodulin antagonist W-7. Autoradiographs show S1 nuclease protection analysis of (a) $\beta 2$ -tubulin, (b) pcf3-21, and (c) pcf8-13 mRNAs. For each panel, time after deflagellation is indicated in minutes above each lane. The graphs show relative changes in mRNA abundance over predeflagellation levels (predeflagellation level is 1). (NDF) Nondeflagellated cells. (d) Kinetics of flagellar outgrowth of cells deflagellated in the presence of W-7. Each SEM is indicated by a vertical line. The flagellar length of nondeflagellated cells (SEM = 0.2) is indicated by the arrow. (open circles with solid line) Cells deflagellated in normal medium; (solid squares with solid line) cells deflagellated in the presence of 25 μ M W-7; (solid squares with dashed line) cells washed out of W-7 into normal medium 120 min after deflagellation.

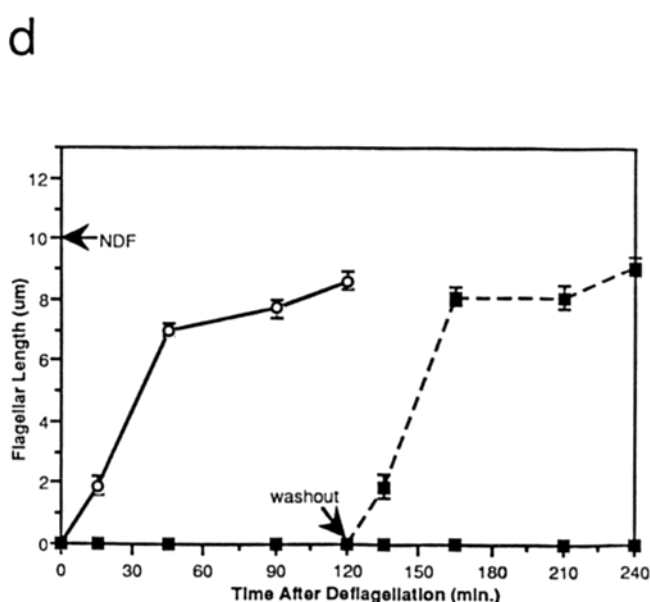
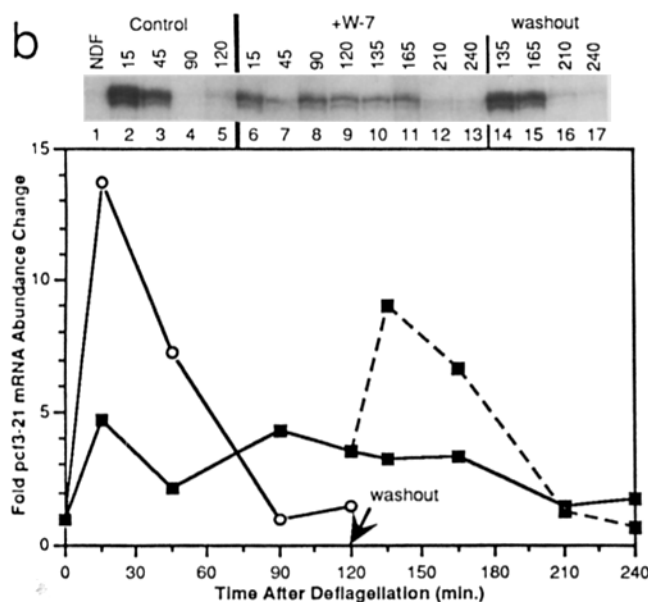
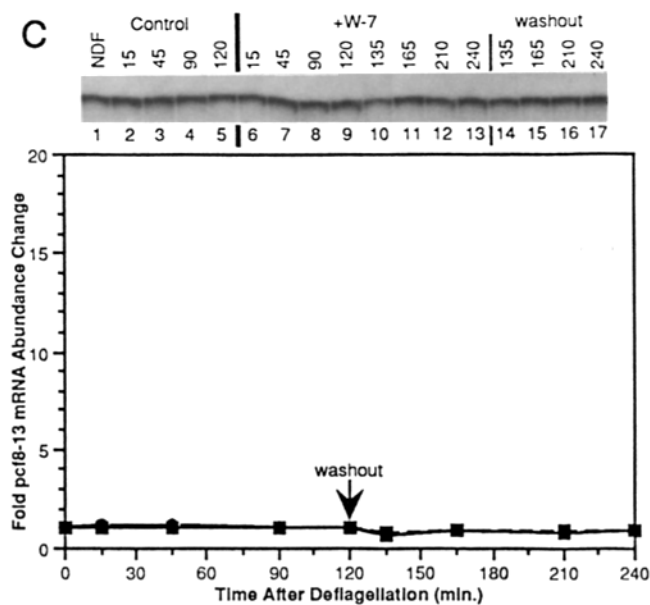
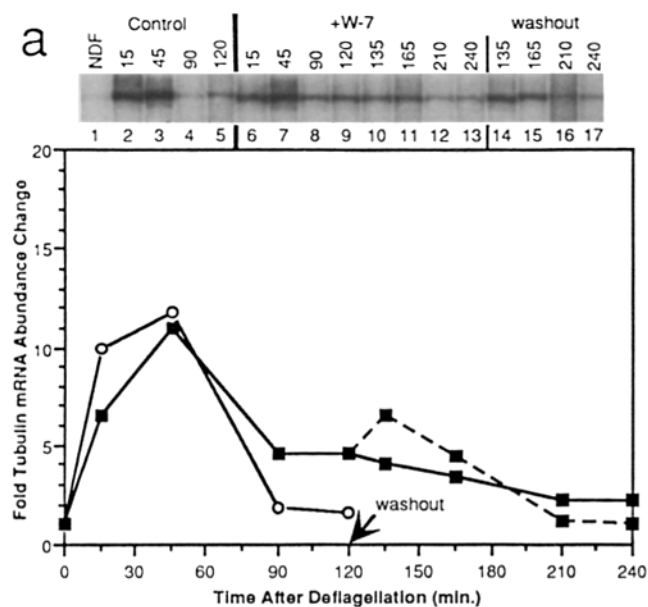
Table I. The Percentage of Cells Deflagellated in Response to Increasing W-7 Concentrations

Concentration of W-7 μM	Minutes after W-7 addition				
	1	3	5	10	30
5	0	0	0	0	0
10	0	1	0	1	0
20	20*	20*	60*	60*	75*
40	100	100	100	100	100

* Also includes cells having lost a single flagellum. The indicated concentrations of W-7 were added to cultures of *Chlamydomonas* cells at 1×10^7 cells/ml. At times after W-7 addition, samples were viewed using phase-contrast microscopy. In addition to eventually causing deflagellation, W-7 also inhibited normal flagellar motility and swimming behavior. Cells began to lose viability after 30 min in 40 μM W-7.

deflagellated without exposure to W-7. Unlike cells held in low Ca^{2+} -containing medium (Fig. 3), cells held in W-7 did not spontaneously initiate flagellar outgrowth even after 120 min postdeflagellation. 25 μM W-5 did not block flagellar outgrowth after deflagellation.

β 2-Tubulin and pcf3-21 mRNA abundance changes occurred in cells deflagellated in the presence of 25 μM W-7 with the same timing as in control cells (lanes 6-9). However, β 2-tubulin and pcf3-21 mRNA levels remained elevated even at 120 min after deflagellation. After W-7 washout at 120 min after deflagellation (lanes 14-17), β 2-tubulin and pcf3-21 mRNA levels increased again within 15 min and then declined to basal levels by 90 min. In these cells, the magnitudes of the β 2-tubulin and pcf3-21 mRNA abundance changes were submaximal. For β 2-tubulin, the initial



peak after deflagellation and the peak that occurred upon W-7 washout were 94 and 54% that of levels in control cells. The corresponding peaks for pcf3-21 were 36 and 64%. Levels of pcf8-13 mRNAs remained constant.

These results using W-7 show striking similarity to the results obtained when the $[Ca^{2+}]_i$ was lowered immediately after deflagellation (Fig. 3). Thus, flagellar outgrowth can be uncoupled from its normal association with flagellar excision and flagellar mRNA abundance changes when cells are deflagellated in the presence of the calmodulin antagonist W-7. These results also suggest that flagellar outgrowth is a Ca^{2+} -calmodulin-mediated process.

Discussion

Ca^{2+} is known to regulate gene expression in few other systems. In mammalian cells, expression of a set of glucose-regulated genes is induced by the calcium ionophore A23187 (20). However, this induction reaches maximal levels rather slowly (after 3-4 h), is sustained, and is blocked by protein synthesis inhibitors, suggesting that induction of these genes may be a secondary effect of elevated Ca^{2+} levels. Ca^{2+} also influences expression and secretion of prolactin and parathyroid hormones in secretory tissues (6, 31). Although part of the rather slow induction of prolactin is due to transcriptional stimulation, Ca^{2+} stimulates prolactin gene expression primarily through a posttranscriptional mechanism that is independent of protein synthesis (17). In the rat PC12 pheochromocytoma cell line, Ca^{2+} fluxes induce rapid, transient transcription of *c-fos* by activating binding of the gene regulatory factor CREB to a specific calcium-responsive DNA element; this induction is independent of protein synthesis (26, 27). Our data introduce the flagellar regeneration system as an important new model for studying Ca^{2+} regulation of gene expression, because in this system the gene induction is rapid, transient, and independent of protein synthesis (1, 2, 9, 25). Also, the gene regulatory response is accompanied by a dramatic morphological change (flagellar outgrowth) that is easy to assay. More importantly, as demonstrated by our work, this system provides the opportunity to examine the interdependence of multiple, overlapping cellular responses to Ca^{2+} signals.

Previous studies in *Chlamydomonas* have shown that an increase in transcription, as well as a transient, posttranscriptional stabilization of flagellar mRNAs, contribute to the normal abundance change after deflagellation (1, 9). Presumably, Ca^{2+} regulates the flagellar mRNA abundance change at one or both of these levels, and further experiments are required to distinguish between these possibilities. We favor the possibility that Ca^{2+} affects flagellar gene expression at a transcriptional level because manipulation of Ca^{2+} affected the time of initiation and the magnitude of flagellar gene expression, but had no major effect on the kinetics of the flagellar mRNA abundance change. Regardless of the level of control, the effects of Ca^{2+} appear to be specific for the set of flagellar genes, because the abundance of a constitutively expressed mRNA was not affected in our experiments. Thus, the flagellar genes are apparently coregulated under these and several other experimental conditions, including pH-shock and mechanical deflagellation, release from resorption conditions, and treatment with herbicides (reviewed in 10).

Our results showing a submaximal mRNA abundance change in response to flagellar excision in low $[Ca^{2+}]_i$ suggest that the normal flagellar mRNA abundance change is a complex response elicited by multiple, normally synchronous controls. Low $[Ca^{2+}]_i$ reversibly blocks a normal abundance change, indicating that one part of the abundance change is regulated by Ca^{2+} . However, additional elements are also required for this response, because the magnitude of the mRNA abundance change occurring upon Ca^{2+} restoration is less than that of control cells (Fig. 2).

Apparently, another control mechanism is associated with the initiation of flagellar outgrowth. In our experiments, a submaximal mRNA abundance change occurs whenever flagellar outgrowth is initiated, either after Ca^{2+} restoration, after W-7 washout, or when cells in a low $[Ca^{2+}]_i$ spontaneously initiate outgrowth. Similarly, Lefebvre et al. (12) showed that when cells are released from resorbing conditions, flagellar mRNA abundance changes occur as flagellar outgrowth initiates. mRNA abundance changes associated with flagellar outgrowth might be controlled by an autoregulatory mechanism that monitors the level of unpolymerized tubulin subunits in the cytoplasm and alters tubulin and flagellar mRNA synthesis or stability in response to a change in the tubulin subunit pool. Such autoregulatory mechanisms controlling tubulin mRNA stability are well characterized in other systems (4). However, conditions exist where the flagellar mRNA abundance change occurs without flagellar outgrowth (Figs. 3 and 4), demonstrating that this correlation is not necessarily causal. Transient, submaximal mRNA abundance changes also occur when cells are deflagellated in the presence of colchicine, a drug that inhibits tubulin polymerization and prevents flagellar outgrowth in *Chlamydomonas* (J. Schloss and L. Keller, unpublished observations). Together, these results indicate that tubulin mRNA abundance changes after flagellar excision can occur independently of flagellar outgrowth and the concomitant decrease in the flagellar protein precursor pool.

Yet another control mechanism apparently requires the normal synchrony of events after deflagellation. In all our experiments, when the $[Ca^{2+}]_i$ is restored to normal levels at 120 min. after flagellar excision, the mRNA abundance change that occurs is less than that of control cells. In these conditions, the $[Ca^{2+}]_i$ is normal (satisfying the Ca^{2+} -requiring control) and flagella initiate outgrowth (satisfying the control associated with flagellar outgrowth), yet the mRNA abundance change is still submaximal. Apparently, elements of this control are stimulated by transient events that occur only immediately after flagellar excision, and are not recapitulated by Ca^{2+} addition. Potentially, this could result from stimulation of alternative signaling pathways related indirectly to the Ca^{2+} requirement or flagellar outgrowth, or the temporal convergence of multiple signals on one of the controls identified above.

Our studies suggest that an intracellular Ca^{2+} flux associated with flagellar loss may initiate a cascade of biochemical and gene regulatory events. This Ca^{2+} flux could occur via Ca^{2+} channels in the cell membrane, intracellular Ca^{2+} release, or through membrane damage caused by flagellar detachment. The latter is not likely, however, since Lefebvre et al. (12) have demonstrated that flagellar gene induction can occur in the absence of flagellar membrane damage. Our results demonstrating a flagellar mRNA abundance

change whenever flagella initiate outgrowth indicate that the signal inducing changes in flagellar gene expression either precedes or is induced by lower $[Ca^{2+}]_i$ s than the signal initiating flagellar outgrowth. A signaling pathway relating these events by concentration and/or time might involve generation of oscillating waves of Ca^{2+} release (15). To examine these possibilities, we have begun measuring intracellular Ca^{2+} fluxes by determining fluorescence changes of Ca^{2+} indicator dyes.

In other systems, phospholipase C, protein kinases, calmodulin, and their targets have been identified as components of Ca^{2+} -dependent signaling pathways. Initial experiments examining the involvement of some of these components in *Chlamydomonas* flagellar regeneration are described here. Although W-7 has been reported to effect molecules other than calmodulin in other systems (3), results presented here suggest that flagellar outgrowth is mediated by Ca^{2+} and calmodulin. In other experiments, we have measured a transient rise in inositol (1, 4, 5) trisphosphate levels within seconds after deflagellation, although flagellar outgrowth and mRNA abundance changes occur even if this rise in IP_3 is blocked by neomycin (19a; R. Crain, J. Cheshire, and L. Keller, unpublished observations). Other signaling components also may be activated during flagellar regeneration in *Chlamydomonas*.

Our ability to temporally uncouple flagellar gene expression and flagellar outgrowth from flagellar excision provides a unique framework for studying Ca^{2+} regulation of gene expression, and the interdependence of Ca^{2+} -regulated cellular processes.

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